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Original Scientific Paper

DOES GALLIC ACID HAVE A POTENTIAL REMEDIAL EFFECT IN EXPERIMENTAL CORROSIVE BURN INJURY TO THE ESOPHAGUS?

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SUMMARY – Gallic acid, acting as an antioxidant, anti-precipitant and cytoprotective agent, was used as a possible remedial natural component for treating experimentally induced esophageal burn. Wistar rats (n=24) were divided into three groups. Control group was given 1 mL 0.9% NaCl. Experimental esophageal burn was induced with 1 mL 40% NaOH application to the esophagus in groups 2 and 3. Gallic acid® (20 mg/kg) was administered to the treated group *via* oral gavage for 10 days. Removed tissues were fixed and paraffin blocks were prepared. Histopathological examination was performed after the sections had been stained with hematoxylin-eosin. Tumor necrosis factor alpha and caspase-3 antibodies were used on immunohistochemical analysis. In the esophageal burn group, necrosis, degeneration and numerous apoptotic cells, as well as intense inflammatory cell infiltration and fibrosis in the muscle layer were observed under light microscope. In the treated group, remodeling of epithelial cells with marked reduction in the connective tissue collagen content was observed, as well as marked reduction in the volume of collagen and abundance of inflammatory cells in blood vessels. Gallic acid treatment may help heal esophageal burns and prevent complications.

Key words: *Esophageal burn; Gallic acid; Tumor necrosis factor alpha; Caspase-3; Immunohistochemistry*

Introduction

Corrosive agents often cause esophageal burns in acute phase, and give rise to narrowing of the esophagus in chronic phase. Corrosives may also burn soft tissues in the oral cavity and stomach. After ingestion of a corrosive substance, perforated esophagus or stomach may be seen within the first weeks[1]. The nature (acidic or alkaline) and amount of the solid or liquid substance ingested determines the type and degree of damage to the digestive system. After the corrosive agent is ingested, esophageal narrowing may arise, along with acute perforation and even death[2]. Various medical agents have been studied for the treatment of esophageal narrowing and management of the healing of esophageal burns[3]. Esophageal burns are generally caused by alkaline intake. Alkaline compounds affect esophageal tissue cell lipoprotein components. Liquefaction necrosis occurs due to severe injury in deep cell layers, such as the muscular layer[4]. The severity of injury also varies depending on the substance pH and tissue exposure time[5,6]. NaOH is often used in experimental models of esophageal burn to investigate aspects of alkaline injury[7]. It is known that free oxygen radical formation and lipid peroxide oxidation occur in esophageal burns, and these initiate tissue injury *via* oxidative stress. When a corrosive substance is ingested, free oxygen radical levels increase within 24 h

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and remain elevated for 72 h in the damaged tissue. In acute corrosive ingestion, free oxygen radicals enhance the increasing tissue damage[8].

The polyhydroxy phenolic compound, gallic acid (3, 4, 5-trihydroxybenzoic acid) can be obtained from grapes, green tea, and other natural sources[9]. It has antioxidant, cytoprotective, and anti-precipitant properties in the gastrointestinal tract[10]. In previous studies, plant and fruit extracts containing gallic acid were demonstrated to have anti-ulcer activity[11].

Cytokine tumor necrosis factor alpha (TNF-α) regulates inflammation and is involved in wound healing *via* tissue reconstruction[12]. TNF-α is responsible for dilation in blood vessels and increasing neutrophil adhesion to the endothelium, thus delivering fluid and leukocytes to the wound[13]. Caspase proteins serve as cysteine protease enzymes in apoptosis. From these family, caspase-3 specifically cleaves many critical cellular proteins. Caspase-3 is involved in apoptosis for chromatin condensation and DNA fragmentation for dissociation of the cell and formation of apoptotic bodies[14]. Reducing the severity of necrosis and inflammation in esophageal burns using a natural antioxidant compound such as gallic acid may support and facilitate burn treatment. This study investigated the possible effects of gallic acid as a remedy in an experimentally induced esophageal burn model of rats *via* examining the histopathological changes and immunoexpressions of TNF-α and caspase-3 proteins.

Material and Methods

Experimental procedure

The study was carried out using Wistar albino healthy female rats (n=24) weighing 200-250 g. Animals were housed in clean plastic cages in conditions of 22±1 °C and 65% humidity at 12/12 h light/dark cycle. The animals were fed standard laboratory chow (commercial standard pellets) *ad libitum*. The experimental protocol was approved by the Ethics Committee of Dicle University Animal Care and Use (decision #2018/8). The standards set in the Guide for the Care and Use of Laboratory Animals (2011, 8th edition) released by the National Research Council were taken into consideration during the experiments. The animals were divided into 3 groups, as follows: group 1 (n=8) as a control group; group 2 (n=8) as esophageal burn group, and group 3 (n=8) as esophageal burn group treated with gallic acid (Gallic Acid®, G7384, Synonym: 3,4,5-trihydroxybenzoic acid, CAS #149-91-7, 97.5-102.5% (titration), Sigma-Aldrich, China). The esophageal burn method described by Sentürk *et al*.[15] was taken as the basis for the model in the present study. Each rat was anesthetized with 50 mg/kg ketamine hydrochloride (Ketalar Flacon, Pfizer, Turkey) and 10 mg/kg xylazine (Rompun, Bayer, Turkey) i.p. Control group was given only 1 mL of NaCl (0.9%). After the anesthesia was maintained, esophageal burn was induced with the help of a 6 Fr Foley catheter. The lumen of the catheter was connected to a balloon to keep the corrosive substance in the esophagus not to pass to the stomach. This method allowed a portion of the esophagus to come in contact with the corrosive substance. The catheter was inserted into the stomach by oral route until the beginning of esophagogastric junction. The esophageal contents were aspirated prior to the application of the corrosive agent, and the experimental esophageal burn was created in the 2 cm distal esophagus by administering 1 mL NaOH (40%) in groups 2 and 3. After waiting for 60 s, NaOH was aspirated. In group 3, after the burn was induced, the first dose of gallic acid® (powder form dissolved in distilled water, 20 mg/kg) was given after 24 hours from the experimental esophageal burn induction, by oral gavage (1 mL each) and the treatment was performed at the same time intervals once a day, and continued for 10 days. During the experimental process, daily observations were made and weight changes in all groups were noted. At the end of day 10, rats were sacrificed under anesthesia and the esophagus was excised.

Histopathological procedure

Excised tissue samples were immediately placed in 10% formaldehyde. After routine histological protocols, tissues were embedded in paraffin blocks and hematoxylin-eosin (H-E) staining was applied to 4-6 μm thick sections which were obtained from paraffin blocks with a microtome (Leica, Germany) and examined under the light microscope (Zeiss Imager A2, Germany).

Immunohistochemical staining

The remaining sections were placed in citrate buffer solution (pH 6.0) for the antigen release procedure two times (8 and 5 min). Sections were washed in distilled water (2x6 min) and H_2O_2 (0.1%, 15 min). In order to reduce nonspecific background staining, Ultra V Block (Invitrogen, US) was applied (10 min). Primary antibodies (TNF-α, lot #ab1793, Abcam,

US [1/100] and caspase-3, lot# ab208161, Abcam, US, [1/200]) were applied overnight. The secondary antibody (Histostain-Plus IHC Detection Kit, cat #85-9073 Invitrogen, UK) (15 min) and streptavidin-peroxidase (15 min) and then to visualize the target antigen, chromogen DAB (3,3'-diaminobenzidine tetrahydrochloride) (DAB Advanced Chromogenic Kit, cat # 8801-4965, Invitrogen, UK) were applied, respectively. Finally, counterstaining was done with hematoxylin and examined under the light microscope (Zeiss Imager A2, Germany) and scored by the histopathologist.

Statistical analysis

GraphPad Prism software (La Jolla, US) was used for statistical analyses and represented as mean ± standard deviation (SD). Histopathological scores were compared and evaluated with Kruskal-Wallis and Mann-Whitney U tests. The level of statistical significance was set at $p<0.05$.

Results

Weight changes

Weight changes were noted in all groups during the experiment and values of all groups were compared, as shown in Table 1. The mean weight of the rats was 223±13 g in the control group and 209±8.7 g in the burn group. There was a significant difference in weight loss between the two groups ($p<0.05$). When the treated group and burn group were compared, the mean weight of the treated group was 216±11 g and it seems that they lost less weight than the burn group ($p>0.05$).

Macroscopic appearance

Macroscopic appearances in each group are shown in Figure 1 a, b, c. Especially in the burn group, esophageal wall was observed to be more edematous and hyperemic than normal.

Table 1. Weight changes in study groups

Group	Mean	SD	SD	p value
Control	223.3750	13.86607	223±13 g	
Esophageal burn	209.8750	8.77395	209±8.7 g	0.031
Esophageal burn + gallic acid	216.0000	11.30107	216±11 g	0.15

SD = standard deviation

1a. Control 1b. Esophageal burn 1c. Esophageal burn + gallic acid

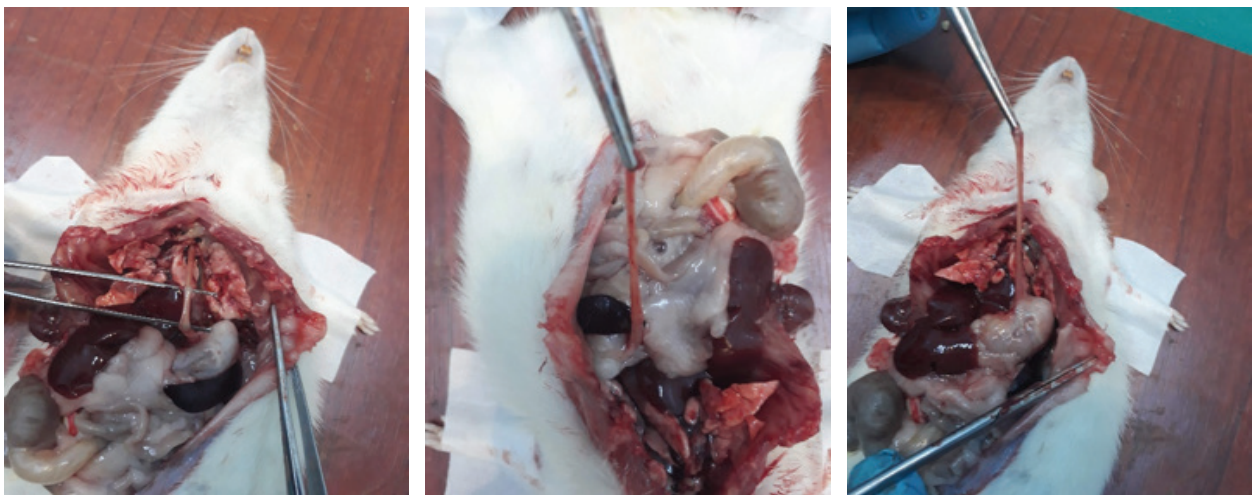


Fig. 1 a, b, c. Macroscopic appearances of study groups.

Histopathological results

Histopathologist counted 10 fields for each criterion. A semi-quantitative histological evaluation scoring system was used to determine histopathological changes. Scoring system using a scale ranging from 0 to 4 was modified from the study by Yukselen *et al*.[16], for instance, epithelial degeneration: 0 (none, no degeneration), 1 (weak, less than 30%), 2 (mild, almost 50%), 3 (moderate, >50%), and 4 (marked, complete degeneration). Collagen deposition was evaluated according to its presence in muscular mucosa, submucosa, and tunica muscularis (Table 2 and Fig. 2).

Control group had no pathological changes. In the mucosal layer, the epithelium was regular but, fibrovascular connective tissue was irregular in lamina propria. Muscle cells were circular and normal in appearance (Fig. 3a). Necrosis and degeneration, besides numerous apoptotic cells, were seen in the epithelium in group 2. Infiltration of inflammatory cells to the epithelium and muscle layer was apparent, as well as collagen deposition, indicating fibrosis

Table 2. Criteria for histopathological evaluation (maximum score is 4)

Criterion	Scoring
Damage to muscular mucosa (epithelial degeneration)	
None	0
Weak	1
Mild	2
Moderate	3
Marked	4
Collagen deposition	
None	0
Weak	1
Mild	2
Moderate	3
Marked	4
Inflammation	
None	0
Weak	1
Mild	2
Moderate	3
Marked	4
Muscle degeneration	
None	0
Weak	1
Mild	2
Moderate	3
Marked	4
Congestion in blood cells	
None	0
Weak	1
Mild	2
Moderate	3
Marked	4

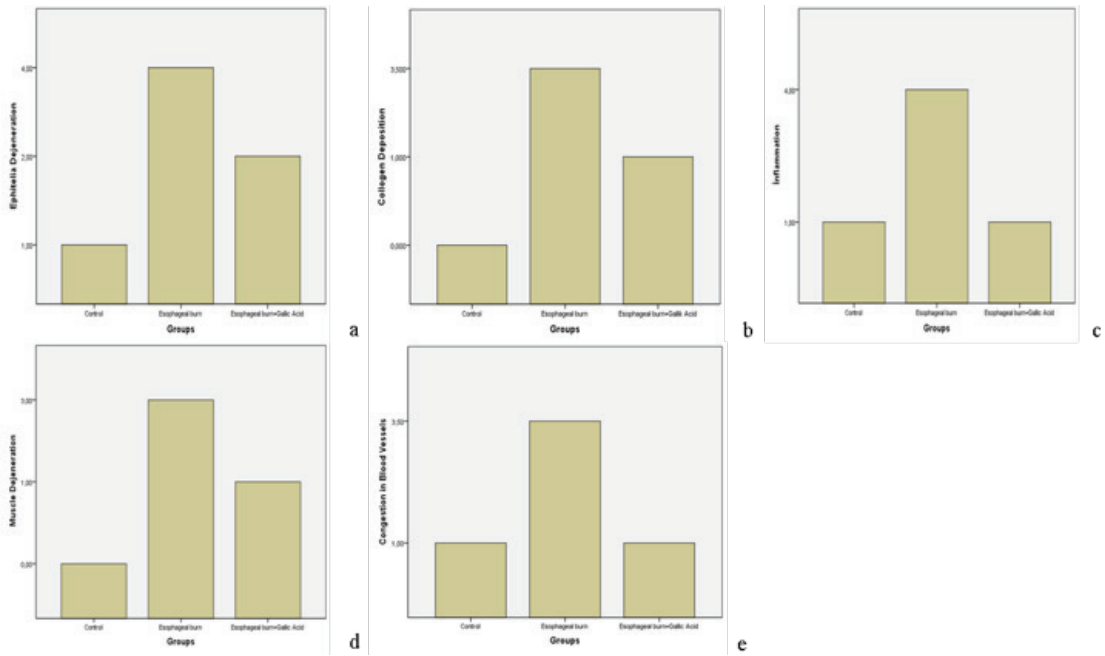
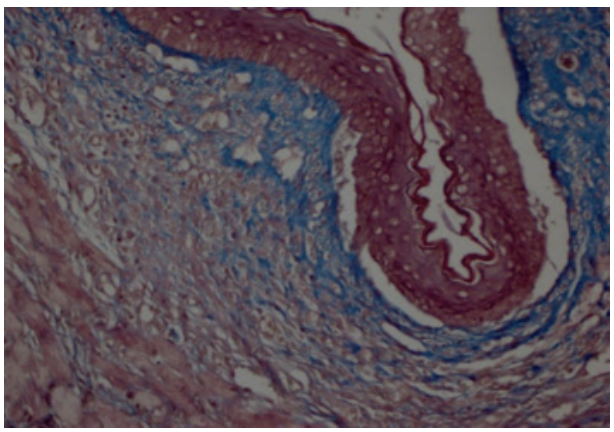


Fig. 2. Damage scoring based on histopathological data.

3a



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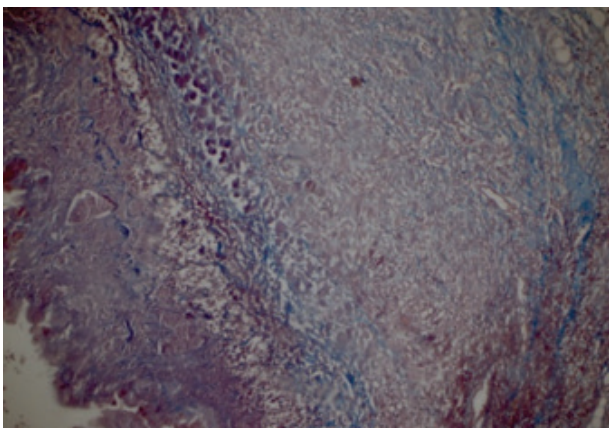

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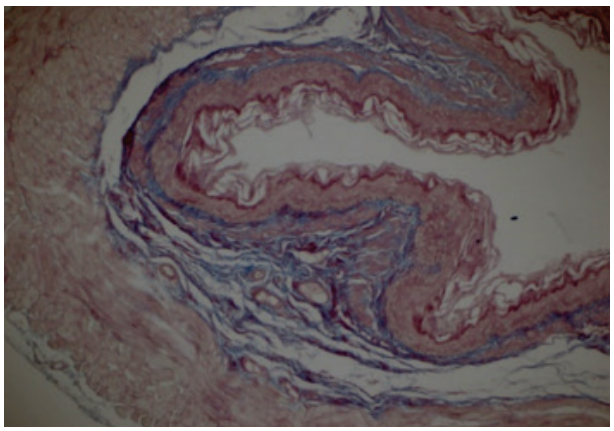
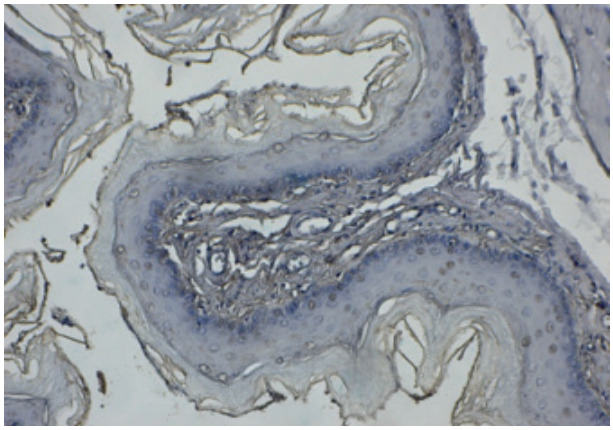


Fig. 3. Histopathological findings: (a) control group: in mucosal layer, epithelium was regular and in lamina propria, fibrovascular connective tissue was irregular. Muscular layer showed ordinary H-E staining, bar=50 μm; (b) esophageal burn group: degeneration, necrosis, and apoptotic cells in the epithelial tissue; scattered inflammatory cells in lamina propria and epithelium; collagen fibers and congestion in blood vessels of the muscle layer. Marked inflammation and degenerative changes in the circular muscle fibers. H-E staining, bar=50 μm; (c) esophageal burn and gallic acid treated group: remodeling in epithelial cells with marked reduction in the collagen unit of the connective tissue, marked reduction in the volume of collagen, inflammatory cells in the blood vessels. Fusiform-shaped cells in the muscle layer with circular distribution. H-E staining, bar=50 μm.

4a



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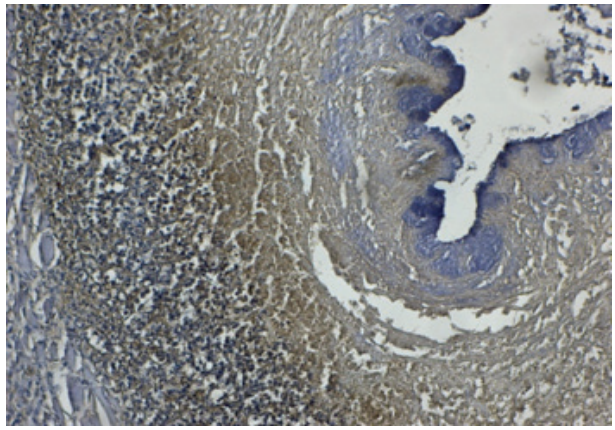

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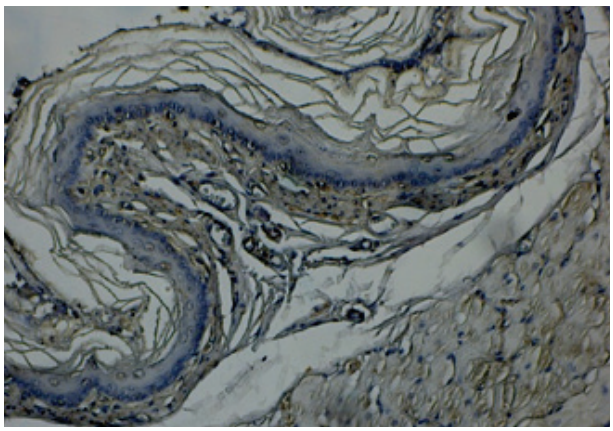


Fig. 4. (a) Control group: negative caspase-3 expression. Caspase-3 immunostaining, bar=50 μm; (b) burn group: caspase-3 expression in blood vessels and depressant endothelial cells in blood vessels, increased caspase-3 expression in intensely infiltrating cells in the connective tissue with prominent epithelial loss. Caspase-3 immunostaining, bar=50 μm; (c) burn and gallic acid treated group: regeneration in epithelial layer, caspase-3 expression in the connective tissue and in solitary disseminated inflammatory cells around the blood vessels. Caspase-3 immunostaining, bar=50 μm.

and congestion in the blood vessels. The burn group also showed an increment of inflammation around the circular muscle fibers with degenerative changes (Fig. 3b). In the gallic acid-treated group, remodeling of epithelial cells, marked reduction in the volume of collagen and in the presence of inflammatory cells in the vessels were observed. The cells in the muscle layer were fusiform and exhibited circular distribution (Fig. 3c).

Immunohistochemical findings

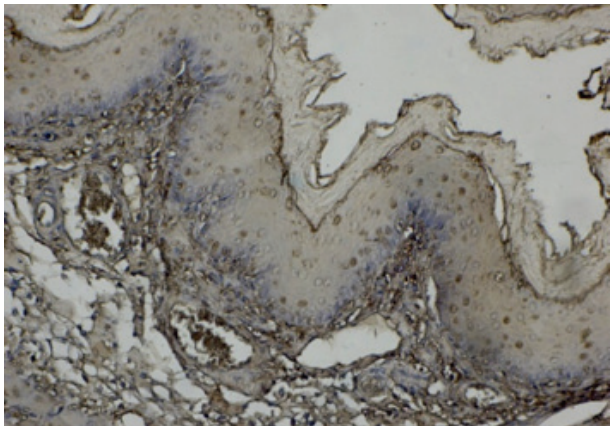
Caspase-3 expression

Control group sections showed negative expression of caspase-3 protein in the whole mucosal layer (Fig. 4a). Caspase-3 protein expression was positive in blood vessels and depressant endothelial cells in the blood vessels of group 2. Caspase-3 expression in the blood vessel endothelial cells was increased along with infiltrating cells of the connective tissue, and prominent epithelial loss (Fig. 4b). In group 3, regeneration of several cells in the epithelium was evident. In the connective tissue, expression of caspase-3 protein was observed in the solitary disseminated inflammatory cells around the blood vessels, as well as in some cells located on the periphery of the muscle layer (Fig. 4c).

TNF-α expression

In the control group, TNF-α was expressed only in the connective tissue macrophages (Fig. 5a). In the muscle layer of group 2, TNF-α expression was increased in macrophages and inflammatory cells of the connective tissue. Also, degeneration was observed in endothelial and inflammatory cells. TNF-α expression was increased in the inflammatory cells between muscle fibers (Fig. 5b). In group 3, positive TNF-α expression was only observed in the inflammatory cells near the basement membrane, macrophages, endothelial cells, and connective tissue cells between the muscles (Fig. 5c).

5a



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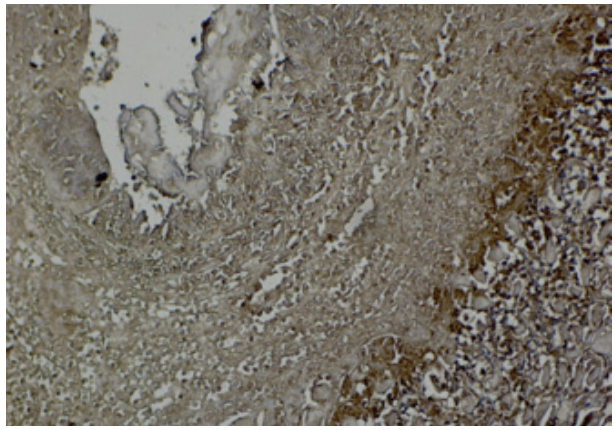

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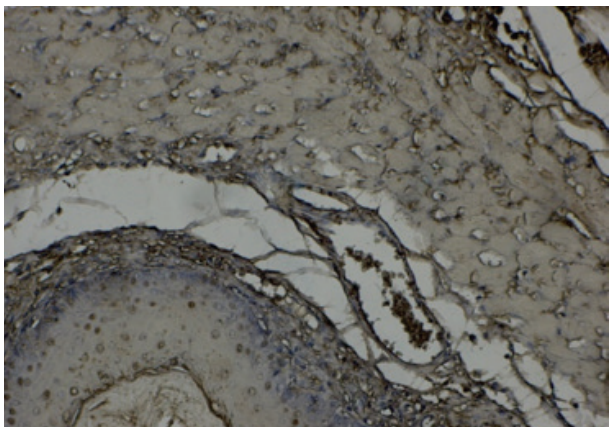

Fig. 5. (a) Control group: TNF-α expression in macrophages of the connective tissue. TNF-α immunostaining, bar=50 μm; (b) burn group: increased TNF-α expression in macrophages and inflammatory cells of the connective tissue; degenerated-congested endothelial cells, and inflammatory cells in the muscle layer. TNF-α immunostaining, bar=50 μm; (c) burn and gallic acid treated group: positive TNF-α expression in the inflammatory cells near the basement membrane, macrophages, blood vessel endothelial cells, and connective tissue cells between the muscles. TNF-α immunostaining, bar=50 μm.

Results of statistical analysis

Comparison of the burn group to the control group revealed inflammation, collagen deposition, epithelial and muscle degeneration, as well as vascular wall congestion to have increased significantly ($p<0.001$). Likewise, all these parameters were significantly improved in the gallic acid treated group as compared to the burn group ($p<0.001$) (Table 3).

Discussion

The status of esophageal burn injury is based on the type of tissue, pH of the caustic substance (acid

Table 3. Statistical analysis of histopathological scoring based on the criteria of inflammation, collagen deposition, epithelial degeneration, congestion in blood vessels and muscle degeneration

	Control	Esophageal burn	Esophageal burn + gallic acid
Inflammation	1.00±0.73$^{\alpha}$	3.84±0.36$^{*,\alpha}$	1.25±0.70*
Collagen deposition	0.37±0.52$^{\alpha}$	3.50±0.54$^{*,\alpha}$	1.00±0.75*
Epithelial degeneration	0.74±0.43$^{\alpha}$	3.73±0.55$^{*,\alpha}$	1.51±0.75*
Congestion in blood vessels	0.76±045$^{\alpha}$	3.36±0.75$^{*,\alpha}$	1.25±0.70*
Muscle degeneration	0.24±0.48$^{\alpha}$	3.13±0.62$^{*\alpha}$	1.00±0.75*

*Significantly different from esophageal burn - esophageal burn + gallic acid groups ($p<0.001$); $^{\alpha}$significantly different from control - esophageal burn groups ($p<0.001$)

or alkali), state of the substance (solid or liquid), and contact time. Acute alkaline ingestion causes liquefaction necrosis in the esophageal mucosa and submucosa. However, in chronic cases, acid ingestion causes coagulation necrosis in the muscular mucosa layer. One of the most important aims of the treatment of esophageal burns is to promote healing of the scar, and another one is to prevent probable stenosis formation[17,18]. Preventive and curative studies on esophageal corrosive burns are actively being conducted, and many alternative compounds have already been applied in experimental models[19,20]. Akbal *et al*.[21] used the herbal extract Ankaferd Blood Stopper (ABS; Ankaferd Health Products Ltd., Istanbul, Turkey) to treat esophageal burn injury and found that this compound prevented inflammation, scar formation, and weight loss. Zeytun and Gokalp Ozkorkmaz[22] applied carvacrol, which many plants have sufficient amounts available, to the experimental esophageal burn injury. Eventually, they observed reduction in fibrosis and apoptosis.

Herek *et al*.[23] noted that ibuprofen was a protective agent against caustic esophageal burn injury in rats, and concluded that treatment with ibuprofen in the acute phase of esophageal burn injury was effective for esophageal healing and decrease in the formation of esophageal stricture. In another study on corrosive esophageal burns, Ozbayoglu *et al*.[24] report on the healing effect of polaprezinc in stenosis prevention. In the study by Ocakci *et al*.[25], ebselen, a synthetic, anti-inflammatory, antioxidant, organoselenium compound, was applied in a caustic esophageal stricture model. The treatment supported the antioxidant system, reduced lipid peroxidation, decreased stenosis index and histopathological damage, and encouraged weight gain compared to esophageal burn group ($p<0.05$). In parallel with this study, we observed weight loss both in the burn and gallic acid treated groups (less than the burn group), giving the opinion that this situation might depend on feeding difficulties because of the burn injury.

Topaloglu *et al*.[26] found that ingestion of a corrosive substance resulted in an acute necrotic phase, with tissue damage that was apparent due to the generation of reactive oxygen radicals. The radicals affected cell membranes and induced accumulation of neutrophils in the affected tissue. Oztan *et al*.[27] report that the esophageal contractile response, which is related to esophageal smooth muscle activity, decreases after experimental caustic burns.

Gallic acid has potent antioxidant capacity, and is an efficient apoptosis-inducing agent that influences cell signaling pathways[28]. Along with its n-alkyl ester derivatives, it has strong inhibitory capacity for myeloperoxidase enzyme activity and scavenging of reactive oxygen species. Herein, we suggest that regular administration of gallic acid after esophageal burn injury seemed to prevent the accumulation of inflammatory cells and decreased collagen accumulation, preventing further necrosis progression in the burn area. This resulted in decreased inflammatory activity and tissue damage in the burn area, inhibiting cell degeneration and apoptosis. We suggest that gallic acid may help prevent stricture development by reducing collagen accumulation in the lamina propria and tunica muscularis (Fig. 3c).

Burn injury is closely related to the immune system suppression and leakage of lymphocytes from the blood and lymphoid organs. After burn injuries, circulating lymphocytes undergo apoptosis. Caspase-3 accelerated in lymphoid cell apoptosis after splenic and burn injuries[29]. Our histopathological and immunohistochemical results recorded in the burn group revealed increased expression of caspase-3 protein in degenerated epithelial cells and connective tissue inflammatory cells. Moreover, we observed caspase-3 activity in inflammatory cells around the vessels, which indicated the presence of intense inflammation and apoptotic process (Fig. 4b).

Tumor necrosis factor alpha, which encourages the caspase cascade and cell apoptosis, is a proinflammatory pleiotropic cytokine[30]. Burn injuries prompt the activation of inflammatory pathways and trigger the release of various cytokines. Experimental evidence suggests that it would be beneficial to observe proinflammatory biomarkers such as TNF-α in these conditions[31,32]. For instance, in alkali-burned cornea tissue, TNF-α is among the cytokines that are upregulated[33]. Ribatti and Crivellato[34] noted that TNF-α is an angiogenic factor, and angiogenic factors promote the proliferation and differentiation of endothelial cells. Connective tissue macrophages are known to have cytokines such as TNF-α, so we observed increased TNF-α expression in macrophages and inflammatory cells of connective tissue in the burn group (Fig. 5b). Furthermore, in the gallic acid-treated esophageal burn group, positive TNF-α expression was observed in inflammatory cells near the basement membrane, macrophages, blood vessel

endothelial cells, and connective tissue cells between muscles (Fig. 5c).

Conclusion

Based on our observations related to the antioxidant and anti-inflammatory characteristics of gallic acid used to treat alkali-induced esophageal burn injuries, we suggest that this compound may have a possible therapeutic effect, at least at the tissue level. However, further research including different techniques is needed to understand the optimal dosage and duration of application.

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Sažetak

IMA LI GALNA KISELINA LJEKOVIT UČINAK KOD EKSPERIMENTALNE KOROZIVNE OPEKLINE JEDNJAKA?

E. Basuguy i *E. Gokalp Ozkorkmaz*

Galna kiselina koja ima antioksidativni, citoprotektivni i antiprecipitirajući učinak primijenjena je kao moguća prirodna ljekovita sastavnica u liječenju eksperimentalno izazvane opekline jednjaka. Wistar štakori (n=24) podijeljeni su u tri skupine. Kontrolna skupina primila je 0,09% NaCl. Eksperimentalna opeklina jednjaka izazvana je nanošenjem 1 mL 40% NaOH na jednjak u 2. i 3. skupini. Galna kiselina® (20 mg/kg) davana je liječenoj skupini putem oralne gavaže kroz 10 dana. Dobiveno tkivo je fiksirano i pripremljeni su parafinski blokovi. Histopatološka analiza provedena je nakon bojenja sekcija hematoksilinom-eozinom. U imunohistokemijskoj analizi primijenjena su protutijela na faktor tumorske nekroze alfa i kaspazu-3. Pregled pod svjetlosnim mikroskopom je pokazao nekrozu, degeneraciju i brojne apoptotske stanice, kao i snažnu infiltraciju upalnih stanica te fibrozu u skupini s opeklinom jednjaka. U liječenoj skupini zabilježeno je remodeliranje epitelnih stanica uza znatno smanjenje sadržaja kolagena u vezivnom tkivu, kao i znatno smanjenje volumena kolagena i broja upalnih stanica u krvnim žilama. Liječenje galnom kiselinom moglo bi biti od pomoći u cijeljenju opeklina jednjaka i sprječavanju komplikacija.

Ključne riječi: *Opeklina jednjaka; Galna kiselina; Faktor tumorske nekroze alfa; Kaspaza-3; Imunohistokemija*